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Synthesis of neoglycopeptides by chemoselective reaction of carbohydrates with peptides containing a novel *N*′-methyl-aminooxy amino acid

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Abstract—A novel *N*′-methyl-aminooxy amino acid has been designed, synthesized, and successfully incorporated into peptides using standard solid-phase peptide synthesis procedures. Reaction of these peptides with native reducing sugars yields neoglycopeptides via a chemoselective reaction with the aminooxy side chains. The key feature of the new amino acid is that it maintains attached sugars in their cyclic conformations and close to the peptide backbone.

Glycopeptides are ubiquitous in nature and play a variety of biological roles.[1] Deciphering these roles in controlled studies requires convenient synthetic access to large numbers of structures. Although many impressive glycopeptide syntheses have been reported, they generally involve complicated techniques and the handling of many sensitive compounds. To greatly reduce the synthetic burden, several groups have turned to chemoselective reactions between completely unprotected peptides and carbohydrates.[2–8] Although the resulting linkages do not precisely replicate natural connections, the simplified procedures allow rapid, convenient access to a wide array of neoglycopeptides, which have been shown by some researchers to maintain their native biological activity.[3]

The most attractive of these chemoselective reaction strategies involves using aminooxy derivatized peptides with natural, unmodified reducing sugars (Fig. 1). However, most published examples have suffered from one of two problems that limit the biological relevance of the product neoglycopeptides: either the aminooxy side chains are extremely long or the attached sugars do not maintain their cyclic conformations. Ideally, neoglycopeptides would have cyclic sugars attached as close as possible to the peptide backbone to allow the sugars to significantly modulate peptide structure and function.

Recently, several research groups have introduced amino acid derivatives that either have short aminooxy

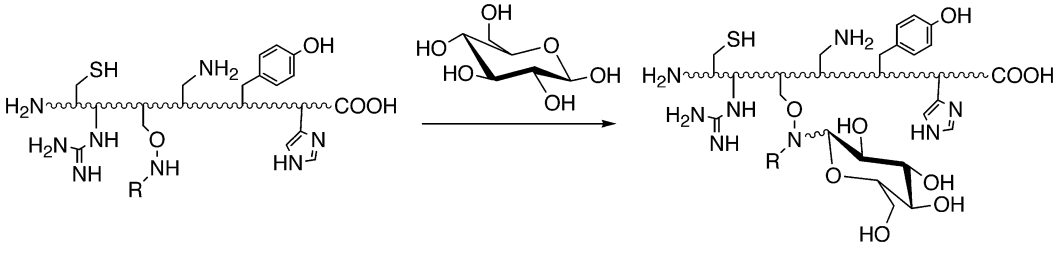

Figure 1. General reaction of aminooxy-derivatized peptides with reducing sugars to form neoglycopeptides. Sugars react chemoselectively with the aminooxy side chains in the presence of a wide variety of other functional groups. When R = alkyl, cyclic sugar conformations are observed, but when R = H, only open-chain oxime-linked sugars are seen.

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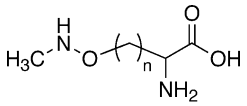
side chains[6] or utilize *N*-alkyl substitution of the aminooxy group to ensure cyclic sugar conformations.[4] We have now constructed a single derivative that combines both of these elements to allow the synthesis of neoglycopeptides with biologically relevant structures.

Our efforts have focused on the two derivatives shown below. These derivatives are the *O*-(*N*-methyl)-amino analogs of serine (**1**) and homoserine (**2**). Both offer the shortest possible amino acid side chains that incorporate the functionality needed for chemoselective attachment of sugars in cyclic conformations.



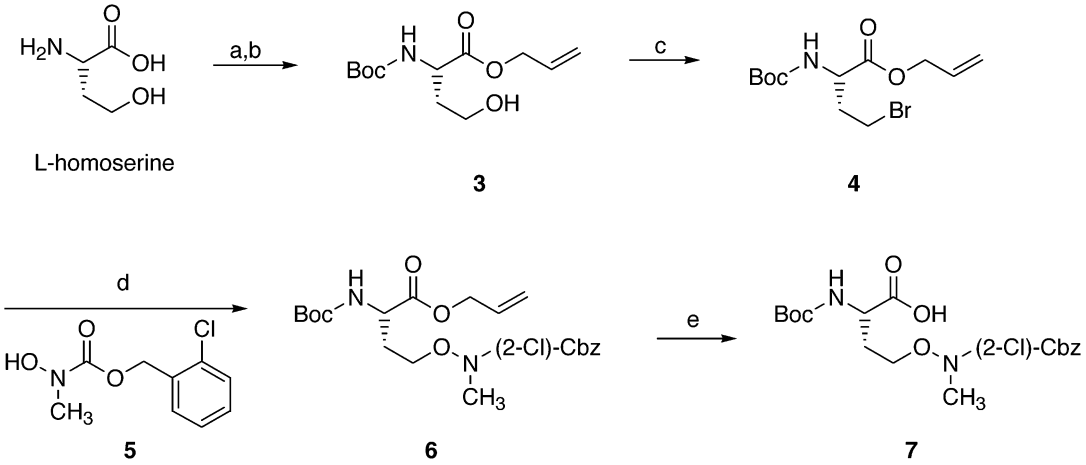
1 : n = 1
2 : n = 2

Initially, we focused on the serine analog **1**, which we successfully synthesized as its *N*-α-Boc, *N*-γ-(2-Cl-Cbz) derivative and incorporated into peptides via standard solid-phase peptide synthesis (SPPS) procedures. Unfortunately, in reactions of these peptides with native reducing sugars, we found that elimination of the *N*-methyl-aminooxy moiety to give dehydroalanine was competitive with the addition of any sugars. In fact, we determined that this elimination occurred spontaneously in aqueous solutions as mild as 0.1 M sodium acetate buffer at pH 5.1. Although our results confirmed the viability of the general strategy, the elimination reaction rendered the derivative impractical for neoglycopeptide syntheses.

We then turned to the homoserine analog **2**, reasoning that the greatly reduced acidity of the carbonyl β protons (relative to the α proton) would restrain any tendency of the aminooxy group to eliminate. This amino acid was conveniently synthesized in its *N*-α-Boc, *N*-δ-(2-Cl-Cbz) protected form from L-homoserine as detailed in Scheme 1.[9] Reaction of L-homoserine with *t*-butyl dicarbonate in basic conditions followed by alkylation of the carboxylate anion with allyl bromide gives the Boc-protected ester **3** in 64% yield for the one-pot, two-step procedure. Subsequent conversion of the alcohol to its mesylate and reaction with lithium bromide yields 88% of the bromide **4**. Displacement of the bromine with the oxy-anion of *N*-methyl-*N*-(2-Cl-Cbz)-hydroxylamine **5** (made by the reaction of *N*-methyl-hydroxylamine with *N*-(2-chlorobenzyloxycarbonyloxy)succinimide) provides the aminooxy ester **6** in 93% yield. Quantitative removal of the allyl ester with catalytic $Pd(Ph_3)_4$ in the presence of pyrrolidine then gives the desired amino acid **7**,[9] suitably protected for Boc-chemistry-based SPPS. The overall yield of **7** from L-homoserine is over 50%, and the procedures are amenable to large-scale synthesis.

We have successfully incorporated **7** into several model peptides (3–9 amino acids each)[10] using standard Boc-chemistry-based SPPS procedures.[11] Each has been deprotected and cleaved from its solid support (trifluoroacetic acid, bromotrimethylsilane, thioanisole)[12] to yield peptides containing the desired *N*-methyl-aminooxy amino acid **2**.[13]

Glycosylation of these peptides proceeds smoothly and chemoselectively in aqueous conditions (0.1 M NaOAc, pH 4.0 or 5.1, 40–45°C) with both D-glucose and lactose. HPLC analysis of a representative glycosylation reaction is shown in Fig. 2. The peptide H_2N–FKAZSK–NH_2, where Z is our aminooxy amino acid **2**, was synthesized as described above, purified by high performance liquid chromatography (HPLC), and characterized by electrospray ionization mass spectrometry (ESI-MS). The lyophilized peptide powder (0.1 mg) was dissolved in 0.1 M NaOAc, pH 4.0 buffer (100 μl), treated with glucose (1 mg), and heated at 40°C. After 26 h, the chromatogram and ESI-MS analysis of the fractions corresponding to the peaks indicated 85% conversion of the parent peptide (peak **A**; calcd for $(M+2H)^{+2}$, 355.4; found, 355.5) to a single, monoglycosylated peptide (peak **B**; calcd for $(M+2H)^{+2}$, 436.5;



Scheme 1. *Reagents and conditions*: (a) $(Boc)_2O$, NaOH, H_2O, CH_3CN; (b) allyl bromide, DMF, 64% from L-homoserine; (c) methanesulfonyl chloride, NEt_3, CH_2Cl_2, then LiBr, acetone, 88%; (d) **5** with NaH, THF, 0°C, then **4**, 93%; (e) $Pd(PPh_3)_4$, PPh_3, pyrrolidine, CH_2Cl_2, 99%.

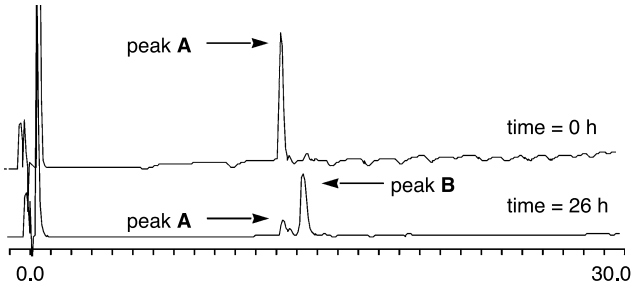


Figure 2. Analytical HPLC chromatograms of the glycosylation reaction between H_2N–FKAZSK–NH_2 and D-glucose in 0.1 M NaOAc, pH 4.0, at 40°C. Peak **A** is the non-glycosylated peptide, and peak **B** is the glycosylated peptide. Samples were run on a Microsorb-MV™ C18 column with a gradient of 0–25% buffer B (CH_3CN+0.08% TFA) in buffer A (H_2O+ 0.1% TFA) over 30 min and monitored at 214 nm.

found, 436.5). Importantly, no additional glycosylation was observed at the N-terminus or lysine or serine side chains,[14] and the glycosylated and non-glycosylated peptides could be separated cleanly by HPLC.

In our various trials, we have found 60–85% glycosylation yields (D-glucose or lactose) after 24–48 h. An equilibrium mixture is reached quickly: the ratio of glycosylated to non-glycosylated peptide does not change significantly after 24 h. As would be expected with an intermolecular process, conversion yields are greatest when the concentration of sugar and peptide are high. In cases of incomplete glycosylation, the non-glycosylated peptide can be recovered, and no peptide or neoglycopeptide decomposition or elimination products have been observed under our reaction conditions.

After isolation, the neoglycopeptides are stable in aqueous solutions at neutral pH. However, at pH 2 they lose their sugars and revert to the parent peptides. We expect that the ability to induce deglycosylation by changing the pH will be useful for potential biological and structural studies.

Our current focus is making neoglycopeptide libraries by reacting series of *N*-methyl-aminooxy-containing peptides combinatorially with a variety of sugars. These libraries are designed to address hypothesized structural and functional roles of glycosylation in biological peptides and proteins. We are also undertaking NMR studies of our neoglycopeptides to confirm cyclic conformations for sugars not previously investigated.[4] Lastly, to extend the use of our strategy, we are synthesizing **2** protected appropriately for Fmoc-based SPPS.

Our new *N*-methyl-aminooxy amino acid derivative **7** can be synthesized in large quantities in very good overall yield. Its incorporation into peptides is accomplished with standard SPPS procedures, and the *N*-methyl-aminooxy side chain provides a selective site for glycosylation under mild conditions. Our methods provide rapid and convenient access to large numbers of neoglycopeptides. By containing cyclic sugars close to the peptide backbone, these neoglycopeptides should serve as excellent mimics of natural glycopeptides. We expect them to enable many new studies of how glycosylation affects the structure and function of biological peptides.

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9. All synthetic intermediates exhibited satisfactory ^1H and ^{13}C NMR and HRMS data. Characterization for **7**: ^1H NMR (400 MHz, $CDCl_3$, 55°C): δ 8.60 (bs, 1H), 7.39 (m, 2H) 7.26 (m, 2H), 5.63 (bs, 1H), 5.29 (s, 2H), 4.43 (bs, 1H), 4.01 (m, 2H), 3.18 (s, 3H), 2.17 (m, 1H), 2.05 (m, 1H), 1.43 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$, 55°C): δ 175.7, 157.6, 156.2, 133.93, 133.90, 130.1, 129.82, 129.80, 127.2, 80.6, 71.0, 65.5, 51.8, 36.7, 30.7, 28.5. FAB-HRMS calcd for $C_{18}H_{26}ClN_2O_7$ $(M+H)^+$: 417.1429; found: 417.1421.
10. Peptide sequences synthesized: Ac–ZAF–NH_2, H_2N–FKAZSK–NH_2, and H_2N–SEZYFLASK–NH_2, where Z is the *N*-methyl-aminooxy amino acid **2**.
11. Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Peptide Protein Res.* **1992**, *40*, 180–193.
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13. All peptides were purified by reversed-phase HPLC and characterized by electrospray ionization mass spectrometry.
14. We have conducted control reactions with H_2N–FKASK–NH_2 under the same glycosylation conditions to verify that no co-eluting mono-glycosylated peptides with a sugar attached at another location were formed. No glycosylation of any kind was observed in the control reactions.